DOCKET NO. TOS-123-USA-C

REMARKS

The present supplemental amendment is made to add a new method claim for the prevention against suppresssion of the antigen presentation function of Langerhans' cells UV irradiation. Support for the present amendment can be found in the Specification on page 15, lines 12-16, and on page 16 of the Specification. The present amendment is deemed not to introduce new matter. Claims 1-4 and 15-18 are in the application.

New Claim 18 sets forth an important aspect of the present invention, namely, the method for the treatment and prevention of suppression of function of Langerhans' cells due to UV irradiation. It was unexpectedly discovered by applicants that when Langerhans' cells were mixed and cultured with T cells obtained by purifying lymph gland cell floating fluid with nylon fiber column, the Langerhans' cells presented the antigen to the T cells and the T cells multiplied (Specification, page 14, lines 24 and 25, and page 15). However, when the antigen was added after irradiating the Langerhans' cells with UV and then the mixed culture with T cells was conducted, a reduction in the multiplication of T cells was observed because the antigen presentation function of the Langerhans' cells was suppressed.

However, when glutathione was added during the ultraviolet light irradiation to study the protective effect of glutathione against the suppression of the antigen presentation function of the Langerhans' cells by UV, it was unexpectedly discovered that

glutathione has a superior immunopotentiating action and effects of alleviating immunosuppression. The results of tests involving the glutathione effect has been plotted in Fig. 1, and a description of this test is set forth in the paragraph bridging pages 15 and 16 of the Specification.

In addition, several of the named inventors of the present invention co-authored an article entitled "UVA-Induced Immune Suppression Through An Oxidative Pathway" in the Society of Investigative Dermatology, Inc., copyright 1999. A copy of this journal article is attached hereto (five pages). This journal article describes the effect of UVA on the antigen presenting function of epidermal cells when measured in terms of antigen-specific T cell proliferation. The tests described in this journal article confirm the unexpected discovery that glutathione may prevent the suppression of the antigen presentation function of Langerhans' cells due to UV irradiation. See, for example, Fig. 7, in the journal article which shows the protective effect of glutathione against UVA.

In view of the data set forth in the Specification and the journal article, it is respectfully submitted that the method now called for in Claim 18 is entirely unexpected and presents a new use of glutathione. For this reason, it is respectfully submitted that new Claim 18 is not anticipated or rendered unpatentably obvious by the references of record.

In view of the foregoing, it is respectfully submitted that

glutathione has a superior immunopotentiating action and effects of alleviating immunosuppression. The results of tests involving the glutathione effect has been plotted in Fig. 1, and a description of this test is set forth in the paragraph bridging pages 15 and 16 of the Specification.

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In view of the data set forth in the Specification and the journal article, it is respectfully submitted that the method now called for in Claim 18 is entirely unexpected and presents a new use of glutathione. For this reason, it is respectfully submitted that new Claim 18 is not anticipated or rendered unpatentably obvious by the references of record.

In view of the foregoing, it is respectfully submitted that

the application is now in condition for allowance, and early action and allowance thereof is accordingly respectfully requested. In the event that there is any reason why the application cannot be allowed at the present time, it is respectfully requested that the Examiner contact the undersigned at the number listed below to resolve any problems.

Respectfully submitted

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UVA-Induced Immune Suppression Through an Oxidative Pathway

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Although ultraviolet B (UVB) irradiation induces local immune or systemic immune suppression, depending on the dose, the immune suppression by ultraviolet A (UVA) has not been fully investigated. In this study, we investigated the effect of UVA on the immune response *in vitro* and *in vivo*. The effect of UVA on the antigen-presenting function of epidermal cells was measured in terms of antigen-specific T cell proliferation. A murine epidermal cell suspension was exposed to UVA *in vitro*, pulsed with trinitrobenzenesulfonic acid, and cultured with T cells prepared from syngeneic mice previously sensitized with trinitrochlorobenzene. UVA (5–20 J per cm^2) suppressed the antigen-presenting function of epidermal cells in a dose-dependent manner, accompanied with suppression of the expression of costimulatory molecules on Langerhans cells. In order to investigate the effect of an antioxidant on the immune suppression, an epidermal cell suspension was irradiated with UVA in the presence or absence of glutathione. The suppressions of antigen-presenting function and ICAM-1 expression were significantly prevented by glutathione in a dose-dependent manner. Further, the effect of UVA on the immune response at the induction phase of contact hypersensitivity was evaluated in terms of lymph node cell proliferation *ex vivo*. UVA irradiation suppressed the endogenous proliferation of lymph node cells in trinitrochlorobenzene-painted mice, and this suppression was significantly reversed by the application of glutathione to the skin during irradiation. These results suggest that UVA-induced immune suppression may be mediated by reactive oxygen species, at least in part. *Key words: antigen presentation/glutathione/Langerhans cells/reactive oxygen species. J Invest Dermatol 112:19–24, 1999*

The exposure of skin to ultraviolet B (UVB) radiation leads to modulation of various biologic processes throughout the whole body. UVB-induced immune suppression is well documented, e.g., in studies on contact hypersensitivity (Noonan *et al*, 1981), delayed-type hypersensitivity (Ullrich *et al*, 1986), and tumor rejection (Kripke, 1986). Acute UVB (100 mJ per cm^2) (Kripke *et al*, 1992) exposure or chronic simulated solar UV (85 J per cm^2) (Ho *et al*, 1991) exposure for 4 wk induced immune suppression *in vivo*. UVB (2.5–20 mJ per cm^2) irradiation *in vitro* induced suppression of Langerhans cell function (Stingl *et al*, 1981; Simon *et al*, 1991; Tang and Udey, 1991; Rattis *et al*, 1995). In addition, susceptibility to UVB-induced immune suppression supposedly has a close relation to the development of skin cancers in humans (Yoshikawa *et al*, 1990). The mechanism of the immune suppression induced by UVB is thought to involve Langerhans cells, which are major antigen-presenting cells that trap antigens in the skin and trigger sequential immunologic events (Toews *et al*, 1980). There have been many studies of the direct effect of UVB (Simon *et al*, 1990, 1991) or the indirect effect of soluble factors secreted from UVB-irradiated keratinocytes (Rivas and Ullrich, 1992; Enk *et al*, 1993) on Langerhans cells. Whereas commercially available sunscreens with a high SPF value can protect the skin from UVB, they are not always protective against UV-induced immune suppression (Ho *et al*, 1992; van Praag *et al*, 1991; Wolf *et al*, 1993). Thus, the effect of ultraviolet A (UVA) that passes through sunscreens on the immune response has attracted interest. It is known that UVA induces DNA damage (Rosen *et al*, 1989), DNA-protein cross-links (Peak *et al*, 1985), and membrane damage (Black, 1987; Tyrrell and Keyse, 1990; Shindo *et al*, 1994); however, the effect of UVA on the immune system is still controversial. Under some experimental conditions, UVA irradiation decreases the number of Langerhans cells in the skin (Alcalay *et al*, 1989; Lavker *et al*, 1995; Grabbe *et al*, 1996). UVA (2.5–20 J per cm^2) irradiation *in vitro* induces suppression of Langerhans cell function (Clement-Lacroix *et al*, 1996). There is another study reporting UVA (46 J per cm^2) induced immune suppression *in vivo* (Bestak and Halliday, 1996), but on the other hand it has been found that UVA has no effect on contact hypersensitivity (Laihia and Jansen, 1994).

Therefore, in order to confirm that UVA can induce immune suppression, we investigated the effect of UVA on the proliferation of T cells as a result of antigen-presenting ability of epidermal cells (Stingl *et al*, 1978; Braathen and Thorsby, 1980). We also measured the expression of cell surface molecules ICAM-1, B7-1, and B7-2 on UVA-irradiated Langerhans cells by flow cytometric analysis, because they are known to play an important role in antigen presentation to T cells (Dang *et al*, 1990; Tang and Udey, 1991; Gaspari *et al*, 1993; Symington *et al*, 1993). Furthermore, UVA is known to produce reactive oxygen species (Shindo *et al*, 1993, 1994). To determine whether immune suppression induced by UVA was due to reactive oxygen species, we investigated the effect of glutathione on the antigen-presenting function of epidermal

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Abbreviations: TNBS, trinitrobenzenesulfonic acid; TNCB, trinitrochlorobenzene.

cells and on ex vivo lymph node cell proliferation (Kimber et al, 1993; Hatao et al, 1994).

MATERIALS AND METHODS

Mice Specific-pathogen-free female C3H/HeN mice were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were used at 8–12 wk of age. The following experiments conform to the guide for the care and use of laboratory in NIH.

Preparation of epidermal cells Epidermal cells were prepared as described before (Singh et al, 1981). Briefly, ears from mice were incubated in a phosphate-buffered saline (GIBCO, Rockville, MD) solution containing 0.5% trypsin (Sigma, St. Louis, MO) for 30 min at 37°C. Epidermal sheets were removed and teased to produce a single cell suspension of epidermal cells in a 0.05% deoxyribonuclease I (Sigma)-RPMI1640 (GIBCO) solution supplemented with 10% heat-inactivated fetal bovine serum (GIBCO).

UVA irradiation of epidermal cells Epidermal cells were suspended in RPMI/10% heat-inactivated fetal bovine serum in a polystyrene dish (2×10^6 cells in 3 ml) and irradiated with UVA through a UVB cut filter (Schott WG335, Shott Glas, Mainz, Germany) in the presence or absence of glutathione (Sigma). The light source was a bank of six FL20S-BLB fluorescent tubes with an emission spectrum of 310–410 nm and a peak at 350 nm (Toshiba, Tokyo, Japan). The irradiance of UVA was about 1.5 mW per cm^2 for in vitro irradiation and 5 mW per cm^2 for in vivo irradiation. The UV intensity was measured with a UVR-3036S Radiometer (Torex, Tokyo, Japan). Sham-treated cell populations served as the control. Cell viability was assessed by trypan blue exclusion.

Antigen pulsing Epidermal cells suspended in RPMI supplemented with 10% heat-inactivated fetal bovine serum were incubated for 30 min at 37°C with 1 mg per ml of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma). After antigen pulsing, cells were washed three times and resuspended in RPMI/10% heat-inactivated fetal bovine serum at a concentration of 1×10^6 cells per ml.

Preparation of T cells Mice were sensitized on their ears with 25 µl of 3% trinitrochlorobenzene (TNCB; Tokyo Kasei, Tokyo, Japan) solution in acetone-olive oil (4:1 vol/vol) for two consecutive days. Four days after the last TNCB treatment, regional lymph nodes (auricular lymph nodes) were excised and disrupted, and the resulting cell suspension was collected. Cells were then passed over a nylon fiber column (Wako, Osaka, Japan). Non-adherent, eluted cells were resuspended at a concentration of 2×10^6 cells per ml. Prepared cells consisted of more than 95% CD4$^+$ or CD8$^+$ T cells, determined by flow cytometric analysis.

Evaluation of the antigen-presenting function of epidermal cells Sensitized T cells (2×10^5 per 100 µl) and antigen-primed epidermal cells (1×10^5 per 100 µl) were mixed and cultured in a 96 well plate for 72 h, as described before (Singh et al, 1981). Eighteen hours before harvesting, 0.5 µCi of [^{3}H]thymidine (Daiichikagakuyakuhin, Tokyo, Japan) was added to each well. Radioactivity of [^{3}H]thymidine incorporated by proliferating cells was measured by a liquid scintillation counter.

Flow cytometric analysis of costimulatory molecules on Langerhans cells After UVA exposure, the cells were collected, washed, and cultured for 24 h. Immediately after and at 24 h after UVA irradiation, the cell-surface molecules were immunostained in PBS supplemented with 1% fetal bovine serum and 0.2% NaN_3 (Sigma). Fluorescein isothiocyanate-conjugated anti-mouse I-A^k monoclonal antibody, phycoerythrin-conjugated anti-mouse CD54 (ICAM-1) monoclonal antibody, biotin-conjugated hamster anti-mouse CD80 (B7-1) monoclonal antibody, biotin-conjugated rat anti-mouse CD86 (B7-2) monoclonal antibody and phycoerythrin-conjugated streptavidin were purchased from Pharmingen (San Diego, CA). Stained cells were washed three times, resuspended, and analyzed by a flow cytometer (EPICS XL Coulter, Hialeah, FL). Langerhans cells were identified as IA^k positive cell subset, and the expression of costimulatory molecules were evaluated by double staining.

Lymph node cell proliferation assay The effect of UVA on the immune system in vivo was evaluated by means of a lymph node cell proliferation assay modified from a reported ex vivo local lymph node assay (Hatao et al, 1994). Briefly, the dorsal hair of the mice was depilated by commercially available hair-remover (Shiseido, Osaka, Japan) and the skin was irradiated with UVA (130 J per cm^2). During the UVA irradiation, 15 µl of 3% glutathione solution in 50% ethanol was topically applied every 2 h. Twenty-four hours after the irradiation, a 3% solution of TNCB was painted on the irradiated sites. Inguinal lymph nodes were excised 3 d after sensitization and disrupted. The resulting cells were washed, resuspended in RPMI/10% heat-inactivated fetal bovine serum at a concentration of 5×10^6 cells per ml, and cultured in a 96 well microtiter plate for 24 h. Eighteen hours before harvesting, 0.5 µCi of [^{3}H]thymidine was added to each well. Endogenous proliferation of the cells was evaluated in terms of the uptake of [^{3}H]thymidine.

Statistical evaluation of results The statistical significance of differences in the means of each data was calculated with Student's t test. Mean differences were considered to be significant at $p < 0.05$.

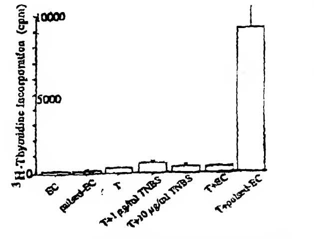


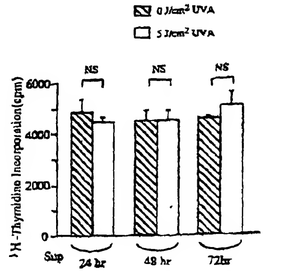
Figure 1. Antigen-pulsed epidermal cells induced proliferation of T cells enriched from lymph node cell suspensions. T cell proliferation was evaluated by [^{3}H]thymidine incorporation. **EC**, intact epidermal cells; **pulsed-EC**, pulsed epidermal cells; **T**, T cell-enriched fraction of lymph node cells from TNCB-sensitized mice; **T + TNBS**, T cell-enriched fraction of lymph node cells from TNCB-sensitized mice with added antigen; **T + EC**, T cell-enriched fraction with epidermal cells; **T + pulsed-EC**, T cell-enriched fraction with pulsed epidermal cells. Data shown are from one representative experiment out of two independent experiments that gave similar results (mean ± SD of triplicate cultures).

RESULTS

Antigen-pulsed epidermal cells induce proliferation of T cells enriched from the suspensions of lymph node cells Intact epidermal cells or pulsed epidermal cells showed a very low [^{3}H]thymidine incorporation. T cell-enriched fraction of lymph node cells from TNCB-sensitized mice with or without TNBS added in the medium did not show proliferation either; however, T cell-enriched fraction cocultured with pulsed epidermal cells showed marked proliferation (Fig 1).

UVA exposure inhibits antigen-presenting function of epidermal cells The proliferation of T cells caused from the antigen presentation by TNBS-pulsed epidermal cells was apparently inhibited by UVA irradiation (0–20 J per cm^2) in a dose-dependent manner (Fig 2). The cytotoxicity was not observed when epidermal cells were irradiated with 2.5–5 J UVA per cm^2; however, 7.5–10 J UVA per cm^2 decreased viability of epidermal cells (Fig 3). Similar suppression was observed when epidermal cells were exposed to UVA either in Hank's balanced salt solution or in RPMI/10% fetal bovine serum without phenol red, suggesting that the major part of the suppression was not due to the phototoxic effect of phenol red under this condition (data not shown).

UVA exposure inhibits upregulation of accessory molecules on Ia-positive Langerhans cell The expression of ICAM-1 on Ia-positive Langerhans cells was increased after 24 h of cell culture, as reported previously (Tang and Udey, 1991). In this series of experiments, UVA irradiation clearly inhibited this upregulation of



...ure 5. Effect of supernatants from UVA-irradiated epidermal ...ls on Langerhans cell function. Intact epidermal cells and T cells ...re cocultured in the supernatants harvested from UVA-irradiated or ...radiated epidermal cells that had been cultured for 1–3 d after irradiation. ...a shown are from one representative experiment out of two independent ...eriments that gave similar results (mean ± SD of triplicate cultures).

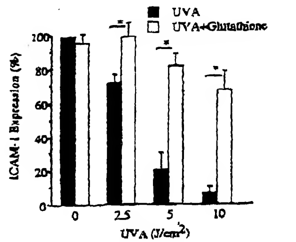
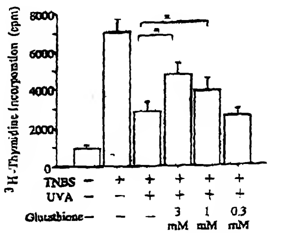


Figure 7. Protective effect of glutathione against UVA (2.5–10 J per cm²)-induced suppression of ICAM-1 expression. Epidermal cells were irradiated with UVA (2.5–10 J per cm²) in the presence or absence of 3 mM glutathione. The ICAM-1 expression (%) was calculated as described in the legend to Fig 2. Data were expressed as the mean ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($p < 0.05$) between the glutathione treated cells and untreated cells.



...ure 6. Protective effect of glutathione against UVA-induced ...pression of Langerhans cell function. Epidermal cells suspended in ...MI/10% fetal bovine serum were irradiated with UVA (5 J per cm²) ...he presence (0.3–3 mM) or absence of glutathione. Data are from one ...resentative experiment out of two independent experiments (mean ± SD of triplicate cultures). The asterisk indicates a statistically significant ...erence ($p < 0.05$) between the glutathione treated cells and untreated ...s.

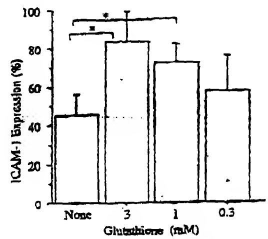


Figure 8. Dose-dependent protective effect of glutathione against UVA-induced suppression of ICAM-1 expression. Epidermal cells were irradiated with UVA (5 J per cm²) in the presence (0.3–3 mM) or absence of glutathione. [The ICAM-1 expression (%) was calculated as described in the legend to Fig 4.] Data are expressed as the mean ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($p < 0.05$) between the glutathione treated and untreated cells.

...J per cm² of UVA was reversed by glutathione in a dose-...endent manner, though 0.3 mM glutathione did not have a ...nificant effect (Fig 8).

...aluation of immune suppression induced by *in vivo* UVA ...adiation At the induction phase of contact hypersensitivity, ...cells proliferate at the regional lymph node upon receiving an ...munogenic signal from antigen-presenting cells. Thus, endogen-...: lymph node cell proliferation has been used for the assessment ...allergenicity of chemicals (Hatao *et al*, 1994). We employed this ...thod to investigate the *in vivo* effect of UVA on the immune ...tem. *In vivo* irradiation with UVA at 130 J per cm² significantly ...reased the endogenous proliferation of inguinal lymph node ...ls prepared 24 h after irradiation. The topical application of ...tathione during the irradiation showed a protective effect (Fig 9).

DISCUSSION

Many studies have shown that UVB suppresses the immune function. Because Langerhans cell plays an important role in the cutaneous immune response, the effect of UVB on Langerhans cell has been extensively investigated (Toews *et al*, 1980; Simon *et al*, 1990, 1991; Enk *et al*, 1993); however, there have been only a few studies regarding the effect of UVA on the immune response (Beasley *et al*, 1996; Bestak and Halliday, 1996). Clement-Lacroix *et al* reported the UVA-induced suppression of allogeneic MELR and observed a protective effect of vitamin E in human epidermal cells *in vitro* (Clement-Lacroix *et al*, 1996). Bestak *et al* suggested that protection against immune suppression by sunscreens is not related to the sun protection factor, but rather to the sunscreens having a broad absorption spectrum (Bestak *et al*, 1995). In this study, epidermal cells from mice were exposed to UVA *in vitro* and

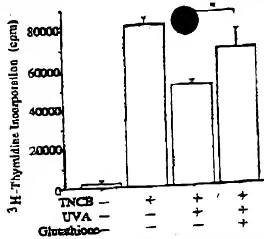


Figure 9. Protective effect of glutathione against the suppression of endogenous proliferation of lymph node cells induced by in situ UVA irradiation. The back of mice was exposed to 130 J per cm² UVA. During irradiation, 15 µl of 3% glutathione solution or vehicle was topically applied every 2 h. TNCB was applied to the irradiated sites on the next day. Radioactivity of [^{3}H]thymidine incorporated by regional lymph node cells was measured 3 d after sensitization. Data are from one representative experiment out of three independent experiments that gave similar results (mean ± SD of triplicate cultures). The asterisk indicates a statistically significant difference ($p < 0.05$) between the glutathione treated cells and untreated cells.

the effects of UVA on antigen-presenting function of epidermal cells and expression of costimulatory molecules on Langerhans cell were investigated. We showed that UVA irradiation dose dependently decreased the ability of epidermal cells to present antigen to T cells directly. This phenomenon was accompanied with suppression of the expression of accessory molecules (ICAM-1, B7-1, B7-2). Thus, it is suggested that the suppression of antigen-presenting function may be due to the suppression of accessory molecule expression. We also showed that 130 J per cm² of UVA irradiation in situ decreased the endogenous proliferation of lymph node cells. These in vitro and in vivo data suggest that UVA suppresses the immune response by modulating Langerhans cell function.

As UVA is known to produce reactive oxygen species, these species may play a role in the phenomena described here. Cells have several natural defense systems against oxidative stress, but they cannot always prevent oxidative stress induced by UVA irradiation (Shindo et al, 1993, 1994). Among the endogenous free radical scavengers, glutathione plays a major role in preventing UVA-induced oxidative stress (Tyrrell and Pidoux, 1986, 1988). Studies with mouse skin showed that cutaneous glutathione rapidly decreased following UVA irradiation (Wheeler et al, 1986; Connor and Wheeler, 1987). We evaluated the effect of exogenous glutathione on UVA-induced immune suppression to establish whether reactive oxygen species are involved.

Suppression of the antigen-presenting function of epidermal cells and the ICAM-1 expression on Langerhans cell induced by UVA (5 J per cm²) was clearly prevented by the addition of glutathione in a dose-dependent manner. Glutathione was included in the culture media only during UVA irradiation, and has no absorption in the UVA range. Also, 5 J per cm² of UVA was not cytotoxic to epidermal cells. These results support the participation of reactive oxygen species in the suppression of the antigen-presenting function of Langerhans cell.

Previous studies have shown that UV induces cellular damage at the plasma membrane, and that subsequent signal transduction activates NF-κB or AP-1 (Devary et al, 1992, 1993). N-Acetyl-cysteine, a precursor of glutathione, added to the cellular medium was transported into the cells and increased the intracellular level of glutathione (Meister 1991). The increased glutathione protected the plasma membrane from UV-induced damage (Devary et al, 1993). In general, [illegible] cytosol to the surrounding medium (Bannai and Tsukeda 1979), but extracellular glutathione is not transported into the cell, except by an Na$^+$-dependent glutathione transport system found in intestine (Linder et al 1984) and kidney (Lash and Jones 1984). In our study, most of the glutathione added to the medium might not have been taken up by epidermal cells, but presumably protected the plasma membrane externally against reactive oxygen species. This hypothesis remains to be confirmed.

In addition to these in vitro experiments, we demonstrated that glutathione applied to the skin partially prevented the UVA-induced immune suppression in vivo. We believe this preventive effect of glutathione should be based on an antioxidative mechanism either at the surface of the skin or inside the skin, because glutathione does not have absorption at UVA range.

Furthermore, in a previous study, vitamin E was found to be effective in reversing the UVA-induced suppression of allogeneic MELR (Clement-Lacroix et al, 1996). As vitamin E is also an antioxidant, it seems likely that antioxidants may generally be effective to prevent UVA-induced immune suppression. Thus, we consider that UVA impairs the immune system at least partially via an oxidative pathway. If this is so, the application of sunscreens containing antioxidants, such as glutathione, should be effective in preventing the immune suppression induced by UV.

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